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TITLE: Cellular Energy Pathways as Novel Targets for the Therapy of Autosomal Dominant Polycystic Kidney Disease

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14. ABSTRACT Autosomal dominant polycystic kidney disease (ADPKD) is a common inherited disorder where patients, over the course of decades, develop large fluid filled cysts that damage the normal kidney tissue and can lead ultimately to kidney failure that necessitates transplantation or dialysis. There are currently no FDA-approved medications for this condition. Recent research reveals that the formation of cysts is due in part both to inappropriate cell growth, fluid secretion, and dysregulation of cellular energy metabolism. The enzyme AMPK regulates a number of cellular pathways, including these disease-causing features. Drugs that activate AMPK, therefore, may constitute an effective therapeutic option for slowing or preventing cyst growth in ADPKD. This research project is aimed at examining the potential of approved, widely used, inexpensive and low-toxicity drugs that can activate AMPK (metformin, simvastatin, and salicylates) and/or promote oxidative metabolism (dichloroacetic acid) as potential therapies for the treatment of ADPKD. During this past research period, we measured and analyzed various metabolomic biomarkers in samples derived from cell lysates and urine and continued collecting cyst growth data in ADPKD <i>in vitro</i> cell culture models and urine specimens derived from a broad cross-section of patients with ADPKD. We also began collecting <i>in vivo</i> data by performing dose-ranging studies of metformin and salicylates in mice.						
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1. INTRODUCTION

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is the most prevalent genetic disease, affecting at least 600,000 Americans. It is characterized by massive cystic growth and enlargement of the kidneys, progressing ultimately to renal failure in ~50% of affected individuals. There are currently no FDA-approved pharmaceutical therapies for ADPKD. The genes associated with ADPKD, *Pkd1* and *Pkd2*, encode polycystin-1 (PC1) and polycystin-2 (PC2), respectively. PC1 and PC2 associate with one another to form an ion channel complex in the cilium and the endoplasmic reticulum whose function and regulation are still poorly understood. Cells that are homozygous for ADPKD-causing mutations are hyper-proliferative and manifest substantial perturbations in energy production. These cells exhibit very high levels of aerobic glycolysis and low levels of oxidative metabolism, reminiscent of the Warburg effect that is seen in tumor cells. The Warburg effect has been recognized as a promising target in efforts to develop new drugs for cancer and other hyper-proliferative diseases. Compounds that force cells to derive their energy primarily from oxidative phosphorylation rather than glycolysis may slow the growth of tumor cells and diminish their capacity to thrive in hypoxic environments such as those that characterize solid tumors and renal cysts. In fact, recent work has shown that administration of 2-deoxyglucose perturbs the dependence of ADPKD cells on aerobic glycolysis and substantially slows cyst growth in mouse models of ADPKD.

The mechanism responsible for this metabolic perturbation has not been elaborated. Very recently, we have identified an entirely novel mechanism that we believe is responsible for this “Warburg-like” excessive glycolytic activity that is observed in ADPKD cells. We have discovered that the PC1/2 ion channel complex interacts with and is regulated by the cellular O₂ sensing machinery. We find that cellular O₂ levels modulate the channel activity of the polycystin proteins, which in turn modulates the level of mitochondrial oxidative metabolism. Pyruvate dehydrogenase (PDH) is a key regulator of aerobic metabolism, transforming pyruvate into acetyl-CoA, which can enter the tricarboxylic acid cycle. Our findings suggest that the activity of PDH constitutes a potential drug target in ADPKD. Dichloroacetic acid (DCA) is a small molecule inhibitor of PDH kinase and thus its administration increases PDH activity. DCA has been used in human clinical trials that tested its utility in treating hereditary lactic acidosis and various forms of cancer. The perturbed energy metabolism in ADPKD results in reduced activity of the AMP-activated protein kinase (AMPK). We have previously shown that activating AMPK with metformin slows cyst growth in *in vitro* and *in vivo* models of ADPKD. Salicylates and statins have been shown to synergize with metformin in activating AMPK. In the studies described in Aim 1 we have proposed to assess the potential of treatment with DCA, metformin, salicylates and statins, alone or in combination, to correct the perturbed energy metabolism in ADPKD and to slow cyst growth *in vitro* and *in vivo*.

One of the current limitations in the development and assessment of new therapies for ADPKD is the lack of clinically useful markers that report on disease severity and progression. The metabolic perturbations that characterize ADPKD-affected cells result in alterations in the inventory of small metabolites that these cells produce. Assessing the profile of metabolites produced by ADPKD cells may lead to the identification of new biomarkers that report on disease progression and on the efficacy of therapeutic interventions. The studies proposed in Aim 2 will seek to identify such metabolism-related markers *in vitro* and *in vivo*, and to determine the extent to which they accurately reflect disease severity in samples obtained from human ADPKD patients. These studies have the potential to identify new drug-targetable pathways that can be exploited in

the development of novel therapies for ADPKD and to identify new biomarkers that may prove to be extremely valuable in future clinical trials. The proposed work also has the potential to be translated rapidly into human clinical studies, as agents that will be examined in the proposed studies have been extensively tested in human clinical trials.

2. KEYWORDS

Autosomal Dominant Polycystic Kidney Disease (ADPKD)

Polycystin-1 (PC1)

Polycystin-2 (PC2)

Metformin

Statins

Salicylates

Dichloroacetic acid (DCA)

AMP-activated Protein Kinase (AMPK)

Biomarkers

Metabolism

Mass Spectrometry

3. ACCOMPLISHMENTS

During the second year of grant support, the Caplan and Hallows laboratories have performed joint studies to measure the dose-response effects of treatment of polarized ADPKD cells with metformin, simvastatin or salicylate on AMPK pathway markers and key metabolomic biomarkers *in vitro*. The Caplan laboratory has further refined conditions for *in vitro* two- and three-dimensional culture of ADPKD cells and, together with the Hallows laboratory, for testing the effects of metformin, statins, salicylates and DCA on their growth and cellular metabolic properties. Additional analysis of various metabolomic biomarkers obtained from human ADPKD urine specimens have also been performed in the Hallows laboratory. Finally, in association with the Somlo laboratory, dose ranging studies have been performed in mice to assess the ability of orally administered metformin and salicylate to active AMPK in renal tissue *in vivo*.

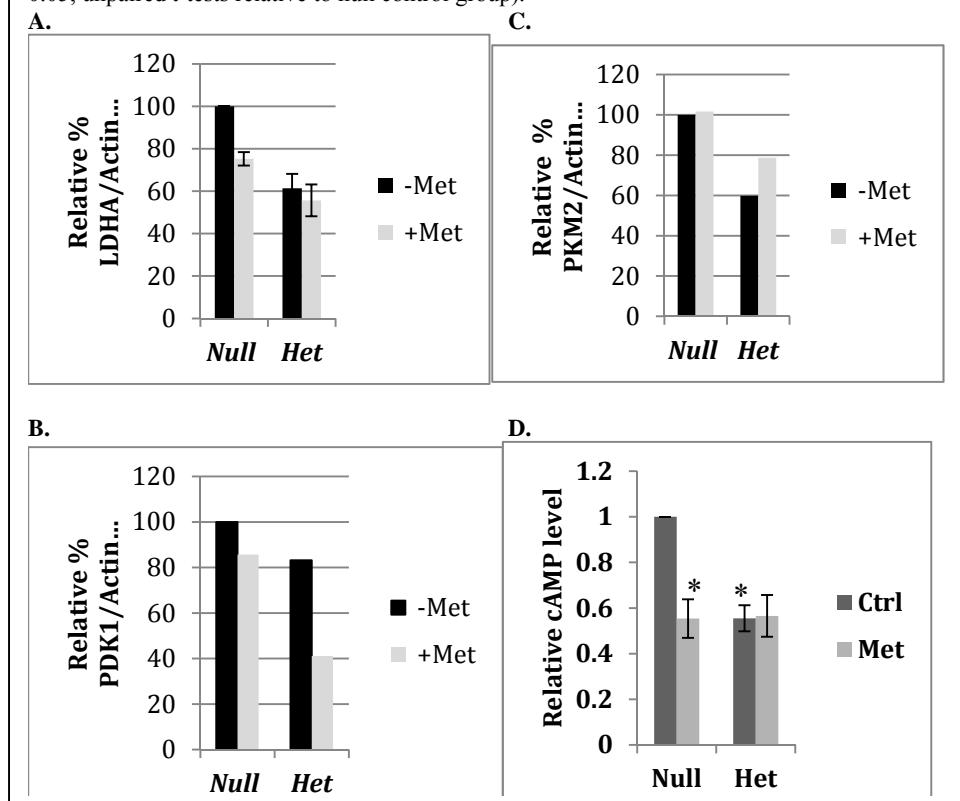
Task 1. Assess whether PDH kinase inhibition or AMPK activation, alone or in combination, corrects the perturbed energy metabolism in ADPKD cells and slows cyst growth *in vitro* and *in vivo*. The studies encompassed in this task group are designed to determine whether and how reducing PDH kinase activity and/or increasing AMPK activity impacts upon the cellular pathways that are involved in cyst development and expansion. These experiments will make use of cultured ADPKD cells and a mouse model of ADPKD to analyze the potential therapeutic utility of reducing PDH kinase activity and/or increasing AMPK activity.

Task 1a: Determine whether treatment with DCA, metformin, salicylate or simvastatin, alone or in combination, correct the perturbed energy metabolism and slow cyst growth in culture models of ADPKD renal cyst formation.

Effects of energy pathway-targeted treatments on aerobic glycolysis in ADPKD cells: We will measure glucose utilization and lactate production using enzymatic assays and mass spectrometry to determine the quantities of these compounds that are present in media that had been bathing cells for various incubation intervals. Dose ranging studies will be performed to identify concentrations of DCA, metformin, salicylate or simvastatin that effectively correct the energy perturbations observed in ADPKD cells. Cell growth and media collection will be performed in the Caplan laboratory; biochemical analysis of the media will be performed by the Hallows laboratory in association with the studies outlined in **2a** and **2b (months 1-8)**.

Research Accomplishments: Initial studies were performed in *Pkd1* -/- (ADPKD) vs. *Pkd1* flox/- (control) cells grown in monolayers on Transwell filters (polarized) and in monolayers in multi-well plates (non-polarized) in the Caplan laboratory. These cells were treated with metformin (1 mM x 24 h) vs. vehicle control. Additional dose response and time course studies of metformin, simvastatin, and salicylates and DCA were carried out on these cells *in vitro* for biomarker analysis along with dose-response growth studies in 3-D Matrigel cultures. Following treatment, lysates from multiple experiments were sent to the Hallows laboratory for targeted metabolomic biomarker analysis (pACC, pp70S6K, pAMPK, pERK, PKM2, PDK1, LDHA, cAMP, lactate, and pyruvate) and to the Pennathur laboratory for preliminary analyses of metabolic intermediates by mass spectrometry (e.g., hexose-6-phosphate, 2,3-diphosphoglycerate, phosphoenolpyruvate, citrate, succinate, and malate).

Figure 1: Effects of cell *Pkd1* KO (null) vs. control (het) and metformin treatment (1 mM x 24 h) in cells grown in vitro on key metabolomic biomarkers. A. Relative LDHA levels as detected by immunoblotting of cell lysates. B. Relative PDK1 levels as detected by immunoblotting of cell lysates. C. Relative PKM2 levels as detected by immunoblotting of cell lysates. D. Relative changes in cAMP levels as detected by enzymatic activity assays (*, $P < 0.05$, unpaired t-tests relative to null control group).



1C). Finally, cAMP levels were significantly higher in the ADPKD cells than in control cells, and metformin treatment significantly reduced cAMP levels in the ADPKD cells (**Fig. 1D**). These results are consistent with the findings of Birnbaum and colleagues, who showed that metformin reduces cAMP production via inhibition of adenylyl cyclase (Miller, RA, et al., *Nature* 494: 256, 2013).

Dose-response studies in *Pkd1*-/- cells *in vitro* demonstrated dose-dependent decreases in LDHA, PDK1, and PKM2 levels and in the enzymatic activities of Erk (pErk/tErk ratio) and PDK1 (pPDHA/tPDHA) with metformin treatment (0, 1, and 5 mM) overnight (**Fig. 2**). Similar dose-response studies using simvastatin (0, 1, and 5 μ M) revealed similar trends in dose-dependent decreases in LDHA, PKM2, pERK/tERK and pPDHA (**Fig. 3**). These findings suggest that the AMPK activators metformin and simvastatin reduce glycolytic capacity and cellular growth in ADPKD cells. The results of these metabolomic biomarker measurements performed in the Hallows laboratory complement studies of 3-D cyst growth rates of these cells in response to the various drugs performed in the Caplan laboratory (**Figs. 4 and 5**). Although there was a reduction of cyst growth rate and circularity of *Pkd1*-/- cells in 3-D culture with salicylate treatment (**Figs. 4 and 5**), there were not significantly altered metabolomic biomarker profiles with salicylate

Of note, our data suggest that LDHA levels are higher in ADPKD (*Pkd1* null) cells than control cells and that metformin treatment reduces LDHA expression in the ADPKD cells (**Fig. 1A**). Similarly, PDK1 expression levels tended to be higher in the ADPKD cells than in control cells, and metformin treatment reduced PDK1 expression (**Fig. 1B**). Although PKM2 levels were higher in ADPKD cells, metformin did not appear to have any significant effect on these levels in our preliminary experiments (**Fig.**

Figure 2: Dose-response effects of metformin treatment (0, 1, and 5 mM overnight) in *Pkdl*-/- cells grown *in vitro* on key metabolomic biomarkers. Relative LDHA (A), PDK1 (B), and PKM2 (C) levels and enzymatic activities of Erk (pErk/tErk ratio) (D) and PDK1 (pPDHA/tPDHA) (E) as detected by immunoblotting of cell lysates. (*, $P < 0.05$, unpaired t-tests relative to 0 mM Ctrl treatment group).

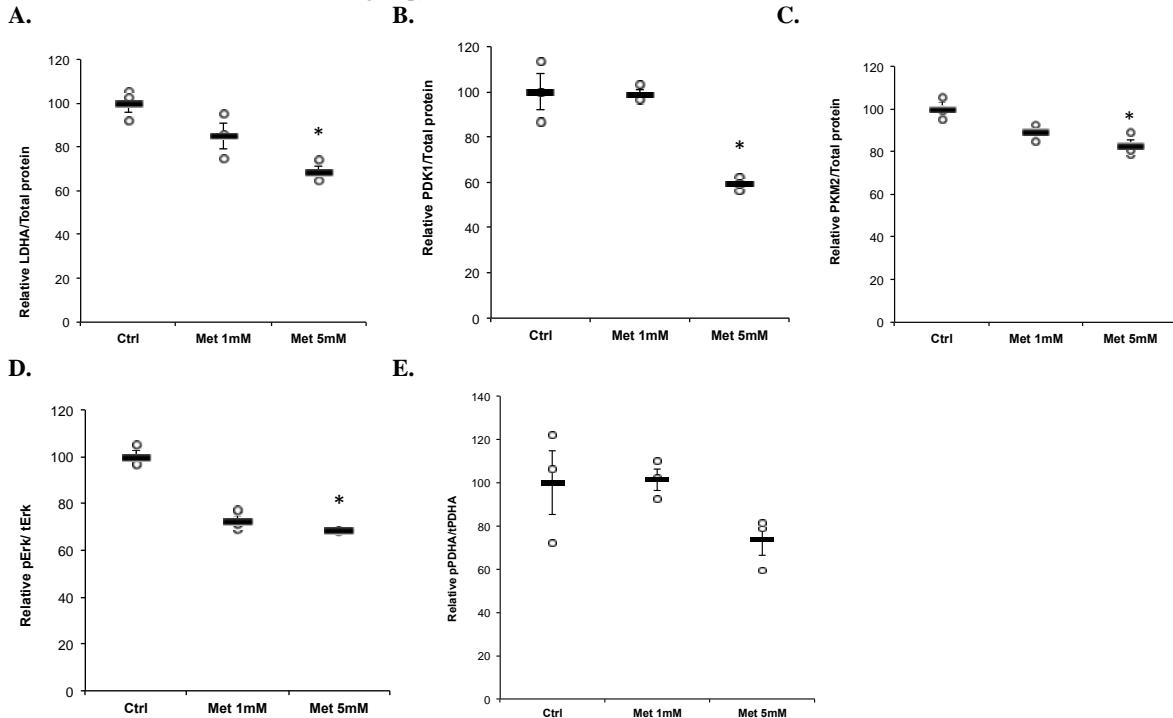
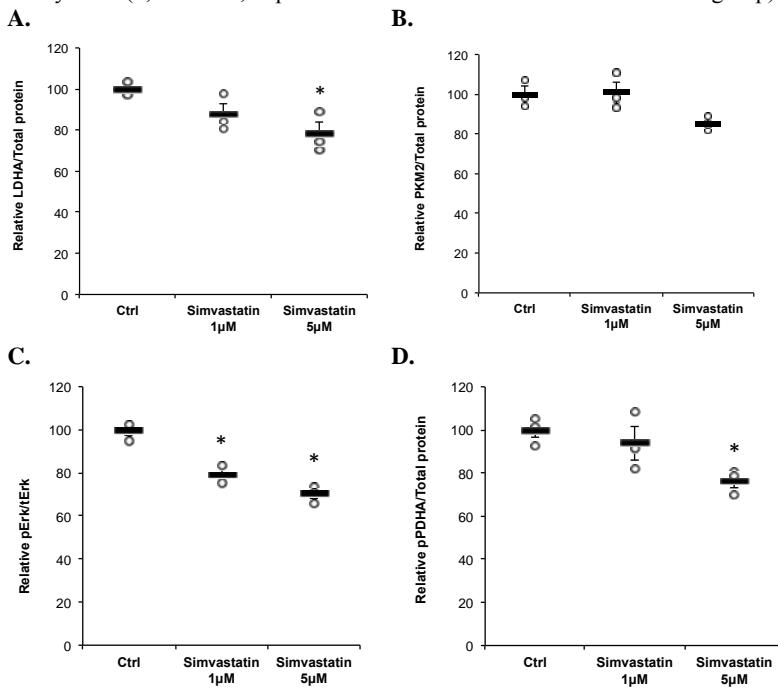


Figure 3: Dose-response effects of simvastatin treatment (0, 1, and 5 μ M overnight) in *Pkdl*-/- cells grown *in vitro* on key metabolomic biomarkers. Relative LDHA (A) and PKM2 (B) levels and enzymatic activities of Erk (pErk/tErk ratio) (C) and PDK1 (pPDHA) (D) as detected by immunoblotting of cell lysates. (*, $P < 0.05$, unpaired t-tests relative to 0 mM Ctrl treatment group).



treatment in preliminary studies performed on these polarized cells grown in culture (not shown). Of note, similar reductions of these biomarkers were found with overnight metformin treatment in *Pkdl*-/- cells obtained from the Baltimore PKD Core Center (not shown).

Additional studies were performed by the Hallows laboratory using the Seahorse XF analyzer system on *Pkdl*-/-, *Pkdl*-/- and control cells to elucidate the effects of genotype and metformin treatment on oxidative metabolism (oxygen consumption rate; OCR) and glycolytic metabolism

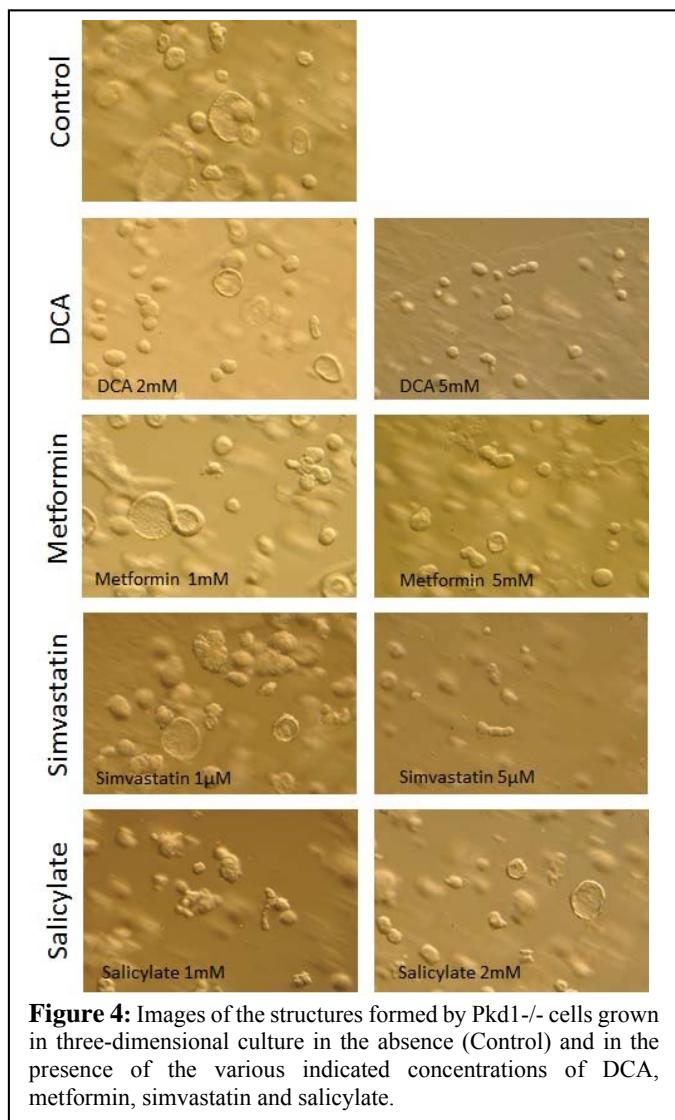
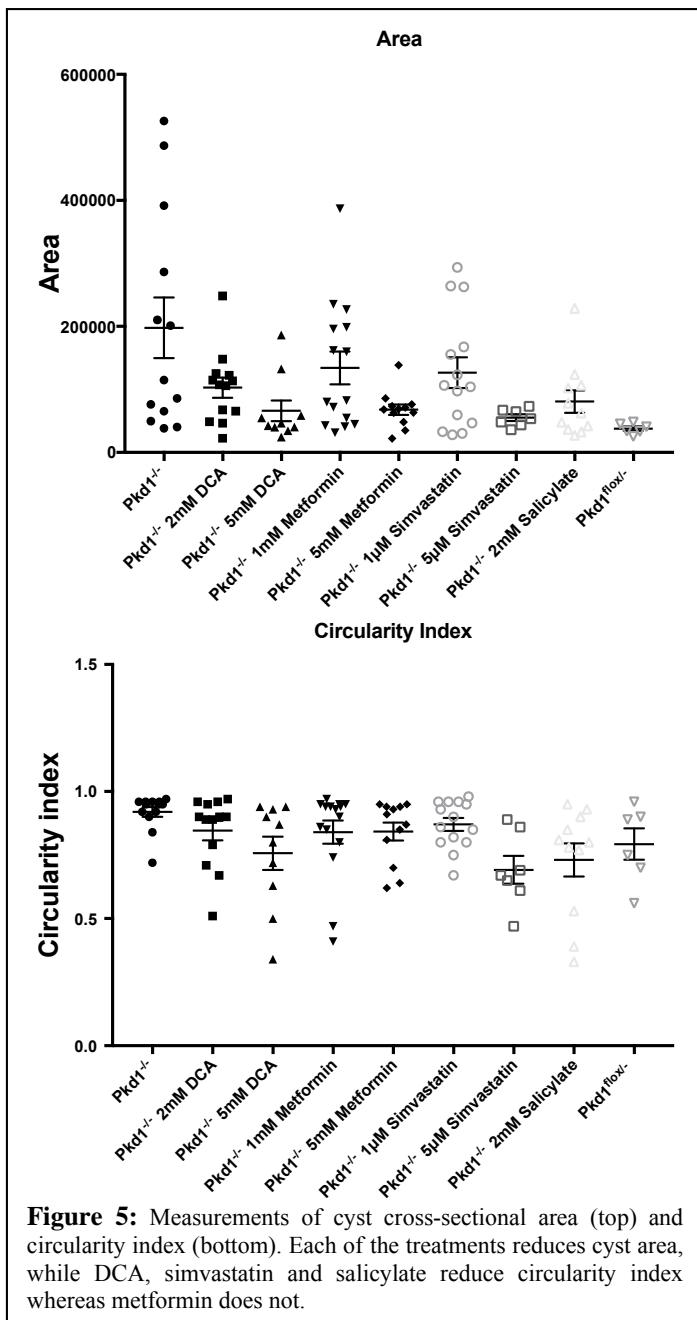


Figure 4: Images of the structures formed by *Pkd1*-/- cells grown in three-dimensional culture in the absence (Control) and in the presence of the various indicated concentrations of DCA, metformin, simvastatin and salicylate.

(extracellular acidification rate; ECAR). Compared with *Pkd1*^{flox/-} control cells, *Pkd1*-/- cells had greater resting OCR and ECAR, indicating increased resting metabolic expenditure in the *Pkd1*-/- cells. However, both cell types exhibited an increase in both OCR and ECAR to comparable levels under maximal stress conditions, indicating that the total oxidative and glycolytic metabolic capacities of control and *Pkd1*-/- cells are comparable (not shown). Treatment of *Pkd1*-/- cells with 1 mM metformin overnight reduced resting OCR and increased resting ECAR. In contrast to the findings in *Pkd1*-/- cells, *Pkd2*-/- cells had similar resting OCR and ECAR relative to control cells, and *Pkd2*-/- cells had a somewhat greater capacity to increase glycolytic relative to oxidative metabolism relative to control cells under maximal stress conditions. In the *Pkd2*-/- cells overnight treatment with 1 mM metformin reduced oxidative capacity significantly and also tended to inhibit glycolytic flux. In summary, metformin treatment consistently reduced resting oxidative metabolism in all cell types, which is consistent with its known inhibitory effect on Complex I of the mitochondrial respiratory chain. In addition, there were somewhat disparate effects on

metabolism seen in *Pkd1*-/- vs. *Pkd2*-/- cells, suggesting that there may be genotypic differences among in their metabolism and cellular bioenergetics profiles. However, these results will need to be confirmed in primary (non-immortalized) cells derived from PKD mouse models as the process of immortalization could artifactually affect cellular bioenergetics.

Effects of PDH kinase inhibition and/or AMPK activation on growth of cultured ADPKD cells: PC1 null (*Pkd1*-/-) and heterozygous (*Pkd1*^{flox/-}) cells will be incorporated into three-dimensional Matrigel matrices, and the size and number of cysts will be determined by quantitative microscopy. Cell proliferation and apoptosis rates will be assessed by immunofluorescence microscopy. The activities of PDH, AMPK and of the mTOR pathway will be determined by Western blotting using phosphorylation-specific antibodies. Media from these cells will be subjected to the metabolomics analyses outlined in **2a** and **2b** to determine whether the doses of DCA, metformin, simvastatin or salicylate that modulate patterns of growth also correct metabolic disturbances. Analysis of cell growth will be performed in the Caplan laboratory, and the Hallows laboratory will analyze culture media in association with the studies outlined in **2a** and **2b** (**months**



6-12).

Research Accomplishments: The Caplan laboratory has continued to test the effects of AMPK activators on the growth of *Pkd1*^{-/-} cells in three-dimensional Matrigel culture. When grown in a three-dimensional collagen matrix, mouse *Pkd1*^{-/-} cells form large fluid-filled spherical cysts, whereas cells that express PC1 at native levels frequently grow in roughly cylindrical “tubule-like” structures. The extent of these morphological differences can be quantitated by using an ImageJ plug to measure the circularity index, which constitutes a ratio in which the magnitude of a structure’s narrowest dimension is divided by the magnitude of the structure’s widest dimension. For a sphere the circularity index will have a value of 1, whereas tubules will have values of less than 1. The Caplan laboratory has previously found that the mean circularity index for the structures formed by *Pkd1*^{-/-} cells is ~0.7, whereas the mean value for cells expressing native levels of PC1 is ~0.5. Importantly, re-expression of the full length PC1 protein in *Pkd1*^{-/-} cells results in the formation of “tubule-like” rather than cystic structures that have a mean circularity index of ~0.5. These observations indicate that the *in vitro* culture system recapitulates quantifiable aspects of cyst formation and that expression of full-length PC1 protein is sufficient to exert anti-cystogenic

activity.

In the first funding year, the Caplan laboratory tested the effects on three-dimensional growth of two concentrations each of DCA, metformin, simvastatin and salicylate. Both circularity index and cyst cross-sectional area were measured. The results of these experiments are depicted in the images presented in **Fig. 4** and quantitated in **Fig. 5**. We found that all of the treatments reduce cyst cross-sectional area in a dose-dependent manner. Furthermore, at the highest concentrations applied, DCA, simvastatin and salicylate reduce cyst circularity index, whereas metformin does not.

During this past year, the Caplan laboratory has focused its efforts on defining the effects on three-dimensional growth of metformin and salicylate. The results of these experiments are depicted in the images presented in **Fig. 6**. We find that both metformin and salicylate dramatically reduce cyst formation when administered in concentrations that should be compatible with those obtainable via *in vivo* delivery. These results motivated the mouse dose ranging studies discussed below in association with **Task 1b**. In addition, the Caplan laboratory has recently obtained immortalized cell lines derived from human ADPKD cysts. The Caplan laboratory is in the process of defining growth conditions that will permit these cells to form cysts in 3-D Matrigel culture.

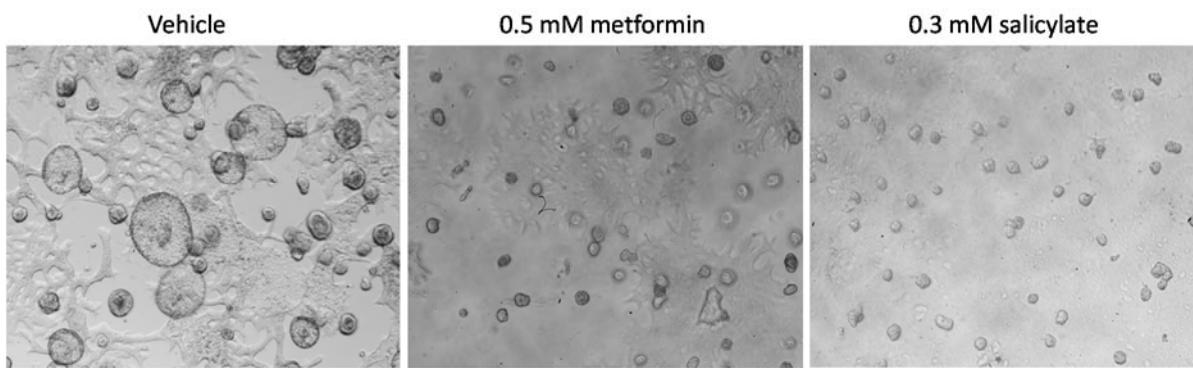


Figure 6: Images of the structures formed by *Pkd1*^{-/-} cells grown in three-dimensional culture in the absence (Control) and in the presence of the indicated concentrations of metformin and salicylate.

Additional 3-D Matrigel cyst growth studies performed in the Hallows laboratory reveal that in *Pkd2*^{-/-} cells (floxed *Pkd2* cells treated with adenoviral Cre recombinase (Ad-Cre)) the cyst growth rate is greater than in control cells (floxed *Pkd2* cells treated with adenoviral empty vector (Ad-Null); **Fig. 7**). Treatment with the cAMP agonists forskolin and IBMX increased cyst growth in both *Pkd2*^{-/-} (Ad-Cre) and control (Ad-Null) cells. Finally, metformin treatment significantly reduced cyst growth rates in both *Pkd2*^{-/-} and control cells both in the presence or absence of treatment with cAMP agonists. In summary, these data indicate that, in addition to its effects on *Pkd1*-dependent cyst growth, metformin also inhibits *Pkd2*-dependent and especially cAMP agonist-dependent cyst growth in 3-D *in vitro* cell cultures.

Planned studies for Task 1a: During the next reporting period, dose response and time course experiments will be completed for metformin, salicylate, and for metformin plus salicylate. This combination was chosen because metformin and salicylate activate AMPK through different mechanisms, and their effects can synergize with one another. Morphometry, cell proliferation and apoptosis rates will be assayed under these conditions, along with metabolomic biomarker analyses. Media samples obtained from cell culture experiments will also be analyzed for biomarker analyses. In addition, optimization of growth conditions for human cyst epithelial cells in 3-D Matrigel culture will be defined. Once these conditions are established, the effects of metformin, salicylate and metformin plus salicylate on cyst formation in the cells will be determined. This analysis will permit the *in vitro* efficacy of these compounds to be assessed in a translationally relevant human cell model system.

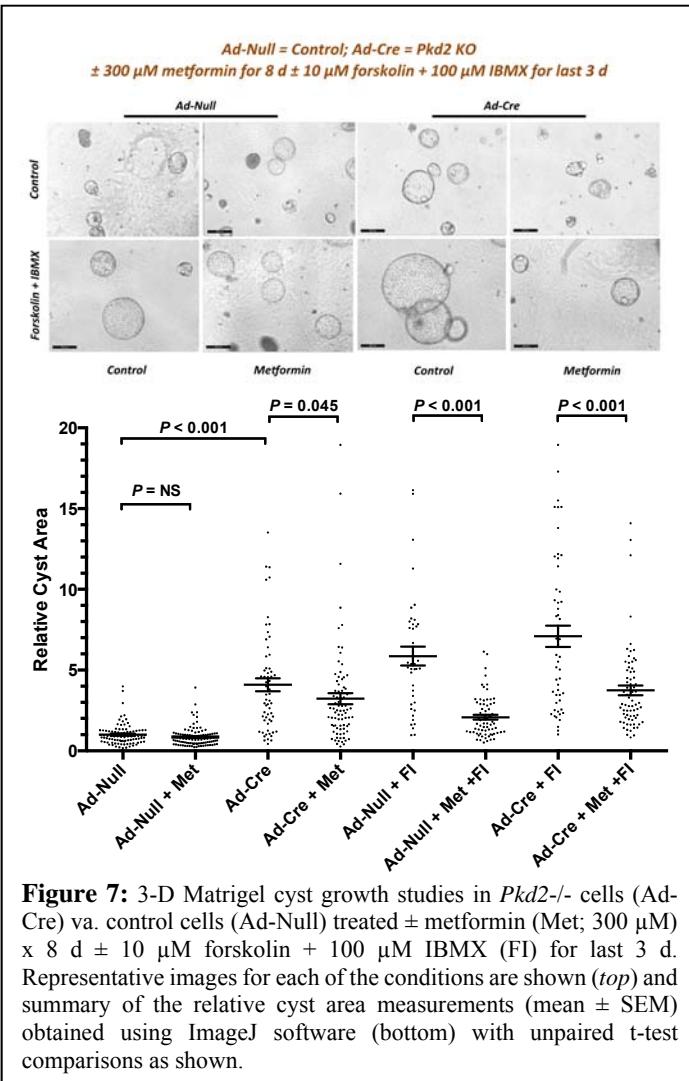


Figure 7: 3-D Matrigel cyst growth studies in *Pkd2*^{-/-} cells (Ad-Cre) va. control cells (Ad-Null) treated ± metformin (Met; 300 µM) x 8 d ± 10 µM forskolin + 100 µM IBMX (Fl) for last 3 d. Representative images for each of the conditions are shown (top) and summary of the relative cyst area measurements (mean ± SEM) obtained using ImageJ software (bottom) with unpaired t-test comparisons as shown.

subset of these mice will be subjected to the metabolomics analyses outlined in **2a** and **2b**. Dose ranging studies will be performed in the Caplan laboratory in association with the Somlo laboratory, while metabolomics studies will be performed in the Hallows laboratory (**months 12-24**).

Assess the efficacy of DCA, metformin, aspirin or simvastatin *in vivo*: *Pkd1*^{flox/-};Pax8 rtTA;Tet-O cre mice will be treated or not (8 mice/treatment group) according to the regimens selected in the studies performed in the previous section. Treatment will begin prior to doxycycline induction and will be carried out for 14 weeks. At the end of the treatment period the mice will be sacrificed and the severity of cyst disease will be determined as outlined in the proposal. We expect to repeat this experiment at least three times. Thus, **at least 120 mice will be required to complete these studies**. Urine and tissue from a subset of these mice will be subjected to the metabolomics analyses outlined in **2a** and **2b**. Treatment and assessment of treatment efficacy will be performed by the Caplan laboratory in association with the Somlo laboratory, while metabolomics studies will be performed in the Hallows laboratory (**months 24-36**).

Task 1b: Determine whether DCA, metformin, aspirin or simvastatin slow cyst growth in a mouse model of ADPKD. (A total of at least 320 mice will be used in these proposed studies, as outlined below)

Defining dose regimens: Prior to assessing the potential utility of DCA, metformin, aspirin or simvastatin administration in *Pkd1*^{flox/-};Pax8 rtTA;Tet-O cre mice, we will establish dose and delivery regimens that are sufficient to inhibit the activity of PDH kinase and/or activate AMPK in the renal tissue of treated mice. Mice (8 per treatment group) will be treated for 7 days, after which they will be sacrificed and their renal tissue will be analyzed by immunofluorescence microscopy or by western blotting. PDH kinase activity will be determined by performing quantitative immunofluorescence microscopy to measure the pPDH/PDH ratio. AMPK activity will be determined by western blot analysis to assess the ratios of pAMPK/AMPK and pACC/ACC. **We anticipate that 200 mice will be required for these experiments.** Urine and tissue from a

Research Accomplishments: In collaboration with the Somlo laboratory, the Caplan laboratory has performed dose ranging studies using orally administered metformin, salicylate, and metformin plus salicylate. Wild type mice were dosed for one week with metformin, salicylate or metformin plus salicylate added to the drinking water. The doses tested and the experimental groups are presented in **Table 1**. Based upon the assumption that an adult mouse drinks about 4 ml/day of

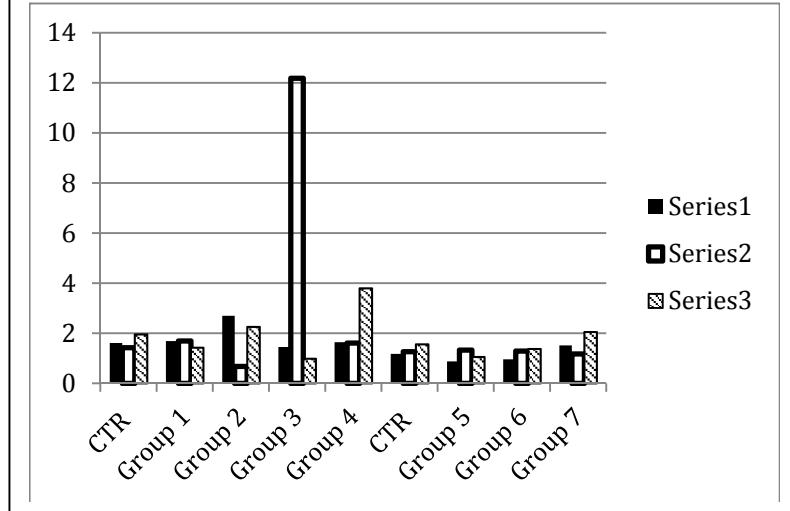
	Metformin mg/kg	Salicylate mg/kg	Number of animals
CTR	/	/	6
Group 1	650	/	3
Group 2	350	/	3
Group 3	50	/	3
Group 4	/	50	3
Group 5	/	10	3
Group 6	650	50	3
Group 7	50	10	3

Table 1: Doses tested in dose ranging study. Indicated drugs were administered in the drinking water.

	Metformin mg/mouse/day (concentration in drinking water)	Salicylate mg/mouse/day (concentration in drinking water)
CTR	/	/
Group 1	13 (3.25 mg/ml)	/
Group 2	7 (1.75 mg/ml)	/
Group 3	1 (0.25 mg/ml)	/
Group 4	/	1 (0.25 mg/ml)
Group 5	/	0.2 (0.05 mg/ml)
Group 6	13 (3.25 mg/ml)	1 (0.25 mg/ml)
Group 7	1 (0.25 mg/ml)	0.2 (0.05 mg/ml)

water and that the average weight of an adult mouse is 20 g, the concentrations of drug that are shown in **Table 2** were added to the drinking water to achieve the desired doses listed in **Table 1**. To mask any unpleasant taste and ensure palatability, 3% sucrose was added to the water. Mice were allowed to drink *ad libitum* for one week, after which their kidneys were harvested and processed for biochemical analysis of AMPK activity. Since even very brief renal ischemia and the concomitant reduction in cellular energy levels is sufficient to induce substantial activation of AMPK, great care must be taken in order to harvest the kidneys in a manner that maintains their circulation until they are frozen. This is accomplished by using specially designed tongs that have been cooled to liquid nitrogen temperatures to freeze clamp the kidneys *in situ* in anesthetized laparatomized mice. The frozen kidneys were homogenized in the presence of phosphatase inhibitors and the constituent proteins in the resulting lysates were separated by SDS-PAGE, electrophoretically transferred to nitrocellulose, and processed for western blotting using antibodies directed against the AMPK α -subunit protein and AMPK α -subunit protein phosphorylated at residue T172 in order to assess the level of AMPK activity. The blots were scanned and the ratio of phospho-AMPK to total AMPK was determined as a surrogate measure of AMPK activity. The data are presented in **Fig. 8**. Each mouse in a dosage group is represented by a separate bar denoted as series 1, 2 and 3. It is evident that there is a high degree of mouse to

Figure 8: Preliminary data depicting relative ratios of pAMPK to total AMPK in the various mouse kidney lysates treated as indicated in Tables 2 and 3.



to activate AMPK in the renal tissue of treated mice. In order to minimize the variability noted above, mice will be singly housed so that the quantity of water and hence drug ingested by each animal can be measured and correlated with the level of AMPK activation achieved. Metabolomics studies pertaining to this work will also be performed by the Hallows laboratory in parallel. Upon completion of the dose ranging study in months 24-28, the Caplan and Somlo laboratories will initiate therapeutic efficacy studies in the orthologous *Pkd1flox/-;Pax8 rtTA;Tet-O cre* mouse model of ADPKD. These studies will take place in months 28-36.

Task 2. Measure candidate metabolomic biomarkers and screen for additional biomarkers that correlate with disease severity and progression in ADPKD mice and cells and that are modulated by reduction of PDH kinase activity or AMPK activation. Cross-correlate biomarker results obtained from mice with banked human urine samples from a cross-section of ADPKD patients (months 0-36).

Task 2a. Investigation of candidate metabolomic biomarkers and profile comparisons as a function of ADPKD severity and treatments.

In vitro studies: Media and cell lysates from experiments outlined in **1a** will be enzymatically assayed for 4 metabolites (lactate, pyruvate, succinate, and cAMP), for 3 glycolytic enzymes (LDHA, PDK1, and PKM2), and for markers of PDH and AMPK activity (total and phosphorylated forms of PDH, AMPK, ACC, and P70S6K). Assays will be performed on triplicate cell lysates from each treatment time point and for each treatment regimen. In addition, media collected over 24-h prior to each time point will also be analyzed. Analysis of metabolites will be performed in the Hallows laboratory, while cell growth and collection will be performed in the Caplan laboratory (**months 1-12**).

Research Accomplishments: See above results outlined under **Task 1a**.

In vivo studies: Urine and renal tissue obtained from animals used in the studies performed in **1b**

mouse variability. In spite of this variability, however, it appears that oral delivery of these agents can induce AMPK activation in renal tissue *in vivo*. In light of these data, the experiment will be repeated under conditions designed to minimize variability, as discussed below.

Planned studies for Task 1b:

During the next reporting period the Caplan laboratory in collaboration with S. Somlo (Co-I) plans to continue and complete experiments to define the relevant *in vivo* dosing regimens sufficient

will be enzymatically assayed for 5 metabolites (lactate, pyruvate, succinate, cAMP, and creatinine), for 3 glycolytic enzymes (LDHA, PDK1, and PKM2), and for markers of PDH and AMPK activity (total and phosphorylated forms of PDH, AMPK, ACC, and P70S6K). Assays will be performed on triplicate tissue homogenates from each treatment time point and for each treatment regimen. In addition, urine collected over 24-h prior to each time point will also be analyzed. Analysis of metabolites will be performed in the Hallows laboratory, while animal treatments and sample collection (**tissue and urine from a total of at least 320 mice; these samples are derived from the same animals employed in Task 1b**) will be performed in the Caplan laboratory in association with the Somlo laboratory (**months 12-36**).

Research Accomplishments: See above results outlined under **Task 1b**.

Planned studies for Task 2a: During the next reporting period, metabolomic biomarker analyses will be performed by the Hallows laboratory for both in vitro cell culture experiments and in vivo kidney tissue homogenates and urine specimens as these samples become available from the Caplan laboratory. In vivo samples will be compared as a function of disease stage in the animals before and after inducible knockout of the *Pkd1* gene by doxycycline treatment. Media samples obtained from cell culture experiments will also be analyzed for biomarker analyses.

Task 2b. Perform broader targeted metabolomic screens by mass spectrometry (MS) to discover novel metabolite biomarkers that may be informative for ADPKD disease severity and response to treatments.

MS analysis to identify new potential markers: The analytes measured in **2a** may be only a subset of the informative biomarkers correlate with ADPKD disease severity, progression, and/or response to therapies. A broad range of metabolic markers will be screened using cell lysate and culture media samples collected *in vitro* (**1a**) and urine and kidney tissue collected *in vivo* (**1b**). The presence of metabolites will be interrogated in targeted platforms by MS, including all glycolytic and TCA cycle intermediates, nucleotides, and amino acids. Metabolite analysis will be performed by the Hallows laboratory with assistance from Dr. S. Pennathur, Michigan Metabolomics and Obesity Center (MMOC) at U. Michigan and statistical consultation from Dr. K. Abebe at U. Pittsburgh. Cell culture and animal-derived samples (**from a total of at least 320 animals-these samples are derived from the same animals employed in Task 1b**) will be provided by the Caplan laboratory (**months 0-36**).

Research Accomplishments: These studies have been initiated with the *in vitro* cell lysate samples described above under **Task 1a** with Dr. S. Pennathur (Co-I: University of Michigan).

Planned studies for Task 2b: During the next reporting period, metabolite analyses by MS will be performed by the Pennathur laboratory for both *in vitro* cell culture experiments and *in vivo* kidney tissue homogenates and urine specimens as these samples become available from the Caplan laboratory. In vivo samples will be compared as a function of disease stage in the animals before and after inducible knockout of the *Pkd1* gene by doxycycline treatment. Media samples obtained from cell culture experiments will also be analyzed for MS analyses.

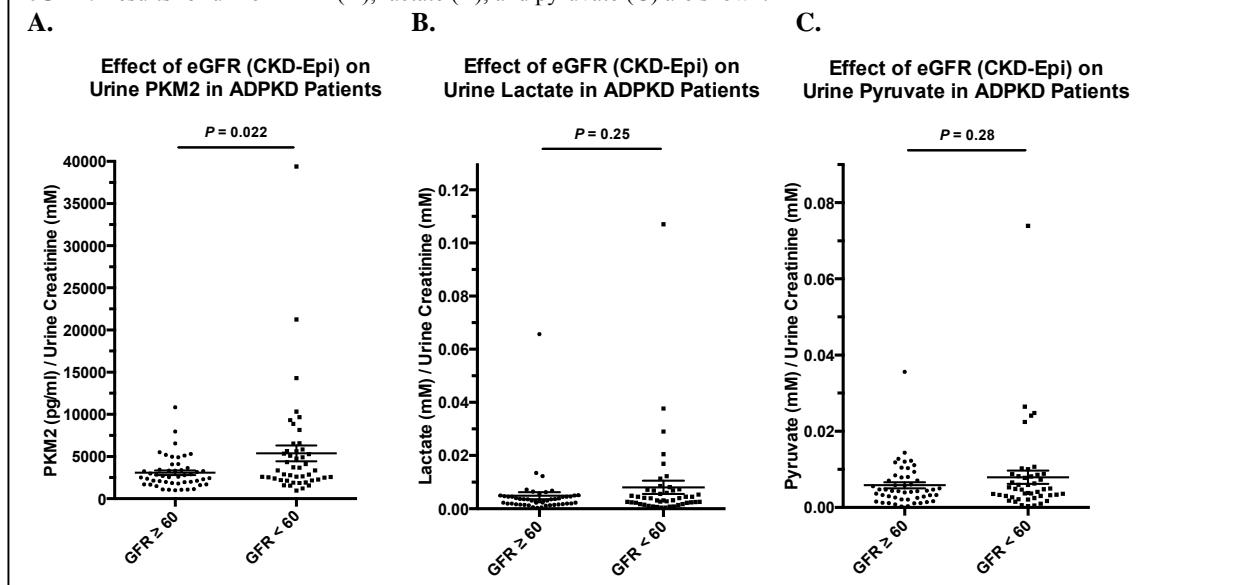
Task 2c. Correlate metabolomic biomarkers from banked ADPKD patient urine samples

with disease severity and progression and compare these results with those obtained in mice.

A subset of the measurements outlined in **2a** and **2b** will be performed on aliquots of patient urine specimens. Metabolite measurements will be correlated with disease severity parameters. **95 banked, coded ADPKD patient urine specimens and clinical data** were provided by Dr. T. Watnick (U. Maryland) and used for this study. Analysis will be performed in the Hallows laboratory with assistance from Drs. S. Pennathur and K. Abebe (**months 20-36**).

Research Accomplishments: Preliminary analyses for all of the 95 banked, coded ADPKD urine samples have been performed by the Hallows laboratory. Although the data are scattered, there was a significant correlation observed with PKM2 levels in urine samples from patients that were stratified by estimated glomerular filtration rate (eGFR) as estimated by CKD-Epi (Tent, H, et al., *Clin J Am Soc Nephrol* 5: 1960, 2010). Specifically, PKM2 levels tended to be higher in patients with more advanced CKD (<60 ml/min; Stage 3 or higher) than in patients with milder CKD (≥ 60 ml/min; Stage 1 or 2) (see **Fig. 9A**). However, urinary lactate or pyruvate concentrations did not appear to stratify as a function of CKD severity in this cross-section of ADPKD patients (**Figs. 9B** and **9C**), nor did many of the other biomarkers tested (not shown).

Figure 9: Preliminary urinary biomarker data in ADPKD patients normalized to urine creatinine and stratified by eGFR. Results for urine PKM2 (A), lactate (B), and pyruvate (C) are shown.



Planned studies for Task 2c: Once additional samples are generated and measurements obtained for the in vivo experiments described above in **Tasks 1b** and **2a**, we will perform correlations of the biomarkers obtained from the banked urine specimens derived from our cross-section of ADPKD patients with those obtained from ADPKD mouse tissue at different stages of disease. Dr. Pennathur will also check a subset of the metabolite measurements on the banked ADPKD patient samples by MS for further correlation once the most relevant ones are identified in the earlier *in vitro* and *in vivo* studies described above. Dr. Abebe (paid consultant) will oversee all of the statistical analyses at the end of the study.

Opportunities for Training and Professional Development Provided by the Project:

Nothing to Report.

Dissemination of Results to Communities of Interest:

An overview of preliminary findings obtained so far in this study were included as part of a 30-minute invited talk by Dr. Hallows at the 2016 Baltimore PKD Symposium that was held on September 19, 2016. His talk was titled “Effects of Metformin on the AMPK Pathway and Metabolomic Biomarkers in ADPKD Kidney Epithelial Cells”. Some of these preliminary results were shared by Dr. Hallows in a poster presentation at the annual ASN Kidney Week meeting held in Chicago, IL in November, 2016 and at the Southern California Kidney Symposium in December, 2016. Dr. Caplan has presented aspects of this work in talks delivered at the FASEB Summer Research Conference on Polycystic Kidney Disease, (Big Sky, Montana) in June, 2017 and at the Western Epithelial Biology Society meeting in March, 2017. In addition, Dr. Caplan visited the Hallows laboratory to conduct an annual face-to-face meeting to discuss the preliminary data obtained by both labs to date, and Dr. Caplan gave a research seminar on aspects of this work at the University of Southern California in March, 2017.

Research Plans During the Next Reporting Period to Accomplish the Goals:

Please see responses above under each individual task.

4. IMPACT

Impact on the development of the principal discipline(s) of the project? The studies performed to date in the Caplan and Hallows laboratories have permitted both groups to establish the experimental parameters that will be required to move the project forward into its next stages. The three-dimensional culture model has been validated, and the conditions that need to be used in the assays of metabolic activity have been established. In addition, initial dose ranging studies have been performed and their methods validated. Repetition of these studies will pave way for the proposed *in vivo* efficacy studies. Thus, the groundwork has been successfully laid for the subsequent phases of the project. These studies have provided tools that will prove extremely useful to the ADPKD research community.

Impact on other disciplines? Nothing to report

Impact on technology transfer? Nothing to report

Impact on society beyond science and technology? Nothing to report

5. CHANGES/PROBLEMS

Nothing to report

6. PRODUCTS

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	Michael Caplan
Project Role:	Initiating PI
Researcher Identifier (e.g., ORCID ID):	NA
Nearest person month worked:	2.4
Contribution to Project:	Dr. Caplan is responsible for co-supervision, with Dr. Hallows, of all of the studies described in the proposal. He is directly involved in every aspect of experimental design and data interpretation.
Funding Support:	NA
 Name:	 Valeria Padovano
Project Role:	Postdoctoral Associate
Researcher Identifier (e.g., ORCID ID):	NA
Nearest person month worked:	12
Contribution to Project:	Dr. Padovano has conducted the in vitro cell growth studies and performed all of the relevant analyses
Funding Support:	NA
 Name:	 Vanathy Rajendran
Project Role:	Research Associate II
Researcher Identifier (e.g., ORCID ID):	NA
Nearest person month worked:	4.8
Contribution to Project:	Ms. Vanathy Rajendran carries out the laboratory's tissue culture work required for the in vitro studies. She also participates in molecular biologic, biochemical and immunocytochemical procedures and maintains laboratory stock solutions.
Funding Support:	NA
 Name:	 Stefan Somlo
Project Role:	Collaborator
Researcher Identifier (e.g., ORCID ID):	NA
Nearest person month worked:	0.36
Contribution to Project:	Dr. Somlo is supervising the breeding of the mouse stocks that will be used in years 2 and 3 of the project.
Funding Support:	NA
 Name:	 Seung Lee
Project Role:	Postdoctoral Associate

Researcher Identifier (e.g., ORCID ID):	NA
Nearest person month worked:	3
Contribution to Project:	Dr. Lee is carrying out the breeding of the mouse stocks that will be used in years 2 and 3 of the project.
Funding Support:	NA
Name:	Kenneth Hallows
Project Role:	Partnering PI
Researcher Identifier (e.g., ORCID ID):	NA
Nearest person month worked:	1.8
Contribution to Project:	Dr. Hallows is responsible for co-supervision, with Dr. Caplan, of all of the studies described in the proposal. He is directly involved in every aspect of experimental design and data interpretation.
Funding Support:	NA
Name:	Hui Li
Project Role:	Senior Investigator
Researcher Identifier (e.g., ORCID ID):	NA
Nearest person month worked:	4.2
Contribution to Project:	Dr. Li has overseen the daily operation of the Hallows lab biomarker measurements and has personally performed all of the immunoblotting and some of the multi-well plate reader studies. He has also performed all of the relevant analyses.
Funding Support:	NA
Name:	Daniel Rivera
Project Role:	Research Lab Technician
Researcher Identifier (e.g., ORCID ID):	NA
Nearest person month worked:	6
Contribution to Project:	Mr. Rivera has conducted most of the multi-well plate reader studies of metabolomic biomarkers on the urine and cell lysate samples along with the relevant analyses.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? Nothing to report

What other organizations were involved as partners? The University of Michigan Metabolomics Core run by Dr. Subramaniam Pennathur (Associate Professor co-investigator on this project) and his team of personnel, including Drs. Jaeman Byun and Lixia Zeng, were

engaged during this past year of support to perform a broader array of analysis of metabolites by mass spectrometry on the experimental samples generated by the Caplan laboratory. They have received a sub-contract awarded through Dr. Hallows' grant award.

8. SPECIAL REPORTING REQUIREMENTS

Collaborative Awards: Reports will be submitted directly by both Dr. Caplan (Initiating PI) and by Dr. Hallows (Partnering PI). These reports indicate the site at which each of the studies described is conducted.